European Journal of Orthodontics 31 (2009) 196–201
doi:10.1093/ejo/cjn095
Advance Access publication 11 December 2008

Myosin proteins identified from masseter muscle using quantitative reverse transcriptase–polymerase chain reaction—a pilot study of the relevance to orthodontics

Archna Suchak, Nigel P. Hunt, Rishma Shah, Andrea C. M. Sinanan, Tim Lloyd and Mark P. Lewis

Divisions of Biomaterials and Tissue Engineering, Craniofacial and Developmental Sciences and Diagnostic and Surgical Sciences, UCL Eastman Dental Institute, London, UK

SUMMARY There is a clearly established relationship between masticatory muscle structure and facial form. Human studies in this area, however, have been limited, especially in consideration of the myosin heavy chain (MyHC) family of contractile proteins. The aim of this pilot study was to assess if differences were detectable between genotype with respect to MyHC isoforms and the vertical facial phenotype in a sample of nine Caucasian female patients, age range 18–49 years, using a novel rapid technique. Masseter muscle biopsies were taken from patients with a range of vertical facial form. The levels of expression of the MyHC isoform genes *MYH 1, 2, 3, 6, 7*, and *8* were compared with the expression in a female calibrator patient aged 23 years with normal vertical facial form, using quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. Statistical analysis was undertaken using Pearson correlation coefficient.

The results showed that there were distinct differences in gene expression between patients with a wide range of variation although changes in *MYH1* were consistent with one cephalometric variable, the maxillo-mandibular angle. The full procedure, from start to finish, can be completed within half a day. Rapid genotyping of patients in this way could reveal important information of relevance to treatment. This technology has potential as a diagnostic and prognostic aid when considering correction of certain malocclusions.

Introduction

The aetiology of malocclusion is a combination of genetic and environmental factors (Proffit, 2000). Studies of families and twins suggest that skeletal pattern and tooth size and number are largely genetically determined (Lundström and McWilliam, 1987). Environmental influences during the growth and development of the face, jaws, and teeth consist chiefly of variable pressures and forces related to physiological muscular activity such as mastication (Kiliaridis, 1995), digit-sucking habits, tongue thrusting, and abnormal breathing patterns (Linder-Aronson, 1979). However, because the soft tissues are attached to the underlying skeletal framework, their effect is also mediated, to some extent, by the skeletal pattern.

Muscles are multicellular contractile units. They are made up of specialized cells that generate motile forces through contraction. There are a variety of myosin isoform genes expressed in mammals (Table 1). The myosin heavy chain (MyHC) isoform protein expression in individual muscle fibres appears to play a defining role in regulating the contractile and histochemical characteristics of a muscle fibre by converting the chemical energy derived from the hydrolysis of adensoine triphosphatase into mechanical force (Weiss *et al.*, 1999; Talmadge, 2000).

Several studies have indicated that masticatory muscles tend to be of reduced volume, and are able to generate less bite force, in patients with a longer vertical facial form (Proffit *et al.*, 1983; Hunt and Cunningham, 1997; Bennington *et al.*, 1999). It has been shown that changes in muscle fibre type and muscle mass can be brought about by switching on one subset and repressing another set of genes to optimize the tissue with respect to power output or fatigue (Goldspink, 2003). Studies have generally shown a consistent change in MyHC isoform switching in response to stretching of the muscle, from initially predominantly fast-contracting variants, to slower, fatigue-resistant types (Talmadge, 2000).

Reverse transcriptase–polymerase chain reaction (RT–PCR) can be used to investigate gene expression, particularly when levels of mRNA are very low. Thus far, the RT–PCR technique has been used principally by groups investigating gene expression in masticatory muscle in animals. Ohnuki *et al.* (2000) used quantitative (q)RT–PCR to investigate changes in masseter-derived mRNA for certain MyHCs and other proteins in rats in response to bite opening. Similarly, Gedrange *et al.* (2001) used the same technique to study the effect of functional advancement of the mandible in pigs, again using the masseter as the focus of investigation. They

Table 1 Myosin heavy chain (MyHC) isoforms investigated in this study.

Gene of interest	MyHC protein	Muscle contraction	GenBank mRNA
MYH1	Myosin heavy chain II x	Fast	AF111785.1 BC114545.1
MYH2	Myosin heavy chain II a	Faster	AF111784.1 BX510904.2 BC126409.1
MYH3	Embryonic myosin heavy chain	Fairly fast	X13988.1 BP232245.1 BQ956249.1
MYH6	Alpha-cardiac myosin heavy chain	Intermediate	D00943.1 BC132667.1
MYH7	Beta-cardiac myosin heavy chain	Slow	M58018.1 X51591.1 AY518538.1 AB209708.1 DQ248310.1 BC112173.1 BC112171.1 EF560725.1
MYH8	Neonatal myosin heavy chain	Fairly fast	X51592.1 Z38133.1 M36769.1

found that the histological findings corresponded favourably with the RT–PCR data. The technique has also been used successfully to analyse myosin isoform changes in developing murine lingual musculature (Maejima *et al.*, 2005). Recently, Gedrange *et al.* (2005, 2006) and Harzer *et al.* (2007) extended their investigations to successfully analyse changes in myosin isoform expression in patients using both RT–PCR and western blotting.

The aim of this pilot study was to examine whether potential differences in MyHC protein expression in masseter muscle biopsy samples of patients with a range of vertical facial morphologies could be identified with a novel system of RT–PCR analysis using sophisticated software and a relative quantification method.

Subjects and methods

This study had the approval of the Eastman Dental Institute and Hospital Joint Research and Ethics committee as part of ongoing departmental research (ref: 00/E014). Signed informed consent was obtained from all patients.

The subjects were nine adult, Caucasian females (age range 18–49 years) undergoing orthodontic treatment and scheduled for orthognathic surgery. They were of good general health and had no known generalized musculoskeletal disorders. The patients selected demonstrated a wide range of vertical skeletal relationships. A 23-year-old female patient with normal vertical facial form, as determined by cephalometric analysis, acted as the calibrator.

Muscle biopsy

Masseter muscle biopsies were obtained under general anaesthesia during orthognathic surgery, or removal of the mandibular third molars in the case of the calibrator patient, following the protocol suggested by Boyd *et al.* (1989). The supero-inferior level of the biopsy was determined by the mandibular occlusal plane, and the tissue was excised from the anterior, deep surface of the masseter adjacent to the anterior aspect of the mandibular ramus. The tissue was obtained via an intraoral incision through the mucosa and buccinator muscle, approximately 3 × 3 × 3 mm (100 mg) excised with a scalpel. All biopsies were obtained by the same surgeon.

Each biopsy was immediately plunged into a TissueProtect tube (Qiagen Ltd., Crawley, West Sussex, UK) containing 2 ml of RNA*later* stabilization reagent (Qiagen Ltd.) to prevent unwanted changes in the gene expression pattern due to RNA degradation or new induction of genes. The samples were incubated at 4°C overnight and then removed from the reagent and transferred to a −80°C freezer for storage.

qRT–PCR

In order to successfully extract RNA from the human masseter muscle samples, the tissue was initially disrupted using the FastPrep FP120A bench top reciprocating device (MP Biomedicals Europe, Illkirch, France). Muscle tissue (weighing > 30 mg) was 'pulverized' in 1 ml of β-mercaptoethanol and 1 ml of lysis buffer (RNeasy Mini Kit, Qiagen Ltd.) against silica ceramic beads (Lysing Matrix D, MP Biomedicals Europe) using the FastPrep device. The resulting solution was processed with the RNeasy Total RNA Isolation Kit (Qiagen Ltd.) and then the resulting RNA quantitated using the 2100 Bioanalyzer system (Agilent Technologies UK Ltd., Wokingham, Berkshire, UK) under nuclease-free conditions. RNA was then converted into cDNA using a high capacity cDNA archive kit (Applied Biosystems, Warrington, Cheshire, UK). Quantitative PCR was performed with these cDNA templates using the TaqMan® System (Applied Biosystems). This system uses specific gene probes conjugated to a FAM (6-Carboxyfluorescein) reporter dye that bind to the sequence of interest (e.g. MyHC-specific transcripts). During the PCR, the probe is cleaved by the action of DNA polymerase that results in increased fluorescence. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye using the ABI Prism® 7300 Sequence Detection System

(All from Applied Biosystems). Relative quantification studies were conducted using an internal control, 18 S rRNA (GenBank X03205.1). Masseter muscle-derived cDNA was targeted for sequences of MyHC genes (Table 1). Data were analysed automatically; briefly, the threshold cycle (C_T) for each PCR was determined as the fractional cycle number at which the fluorescence generated by the reaction exceeded that due to background fluorescence. This was then used to determine the expression levels of each gene. Basically, the mean C_T values for triplicate repeats of the PCR for both the gene of interest and the internal control (18 S rRNA) were determined and the difference between the two was calculated [e.g. if C_T for 18 S rRNA is 10 and the C_T for *MYH1* is 15 then the difference (ΔC_T) is 5]. The same process is repeated for a calibrator sample; in the present study, a 23-year-old female patient with normal vertical facial form was used. The ΔC_T for the calibrator is subtracted from the ΔC_T for the patient of interest and then converted into a ratio by the formula $2^{-\Delta\Delta CT}$. These values were plotted against the maxillo-mandibular (MM) angle using Microsoft Excel and the Pearson correlation coefficient and unadjusted *P*-value calculated using the same system.

Cephalometric analysis

All patients had a lateral cephalogram taken as part of their ongoing treatment. Vertical facial dimensions were recorded based upon measurements of the MM angle and lower anterior face height (LAFH) expressed as a percentage of total anterior face height (British Standards Classification, 1983).

Results

Patient sample

The calibrator patient exhibited a MM angle of 27 degrees and a LAFH ratio of 55 per cent. The nine 'test' patients demonstrated a range of MM angles from 17 to 47 degrees and a LAFH% from 52 to 60 per cent.

Confirmation of gene expression

Isolation of RNA from the highly fibrous masseter muscle, using the FastPrep device was possible. The properties of this tissue require extensive periods of homogenization that generate significant amounts of heat which can lead to severe degradation of the nucleic acids. The technique described yielded good-quality RNA for RT–PCR analysis as shown in Figure 1. This graphical quality control output from the 2100 Bioanalyzer shows a nucleic acid size ladder (L) alongside five representative RNA samples. The major 18 S and 28 S rRNA bands were clear and distinct and the lanes contained no smearing or other spurious bands that are indicative of sample degradation.

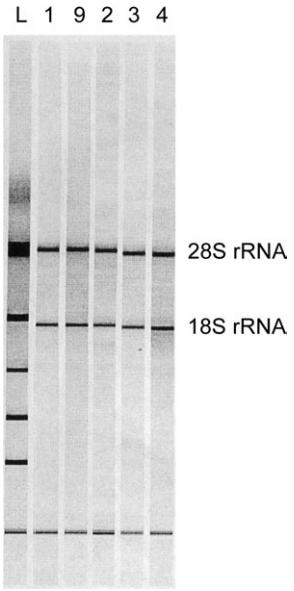

Figure 1 Results of capillary electrophoresis of RNA samples. L contains an RNA sizing ladder. 1, 2, 3, 4, and 9 are representative patient samples. The major 18 S and 28 S rRNA bands are clear and distinct and the lanes contain no smearing or other spurious bands that are indicative of sample degradation.

Relative gene expression between patients

Using the high-quality isolated RNA, transcripts of all MyHC isoforms were detectable by qRT–PCR in all patient samples studied. The fidelity of the PCRs was confirmed by amplification curves as shown in Figure 2. These data are from the samples of one patient and show the tight clustering of the curves for each of the genes shown on this trace (the internal control, 18 S and the *MHY 1*, *2*, and *3*). The abundance of a transcript is determined by the cycle number at which the transcript first appears [where the line crosses the horizontal (*x*)-axis]; therefore, in the representative sample shown, the abundance was 18 S > *MYH2* > *MYH1* > *MYH3*. Relative quantification uses these cycle numbers to determine the change in expression of a target nucleic acid sequence in a test sample relative to the same sequence in a calibrator or reference sample. The targets in all cases were the probed sequences for each MyHC isoform. The variation of expression relative to the patient's MM angle is shown in Figure 3. Of particular note was the strong negative correlation between *MYH1* and MM angle (Pearson's correlation coefficient $r = -0.7338$; unadjusted *P*-value = 0.0244). There was also evidence of clustering with *MYH3* (embryonic MyHC) but the significance of this observation is unknown.

Discussion

This appears, to the best of our knowledge, to be the only human study to investigate the relationship between expression of masseter MyHCs and vertical facial form using this

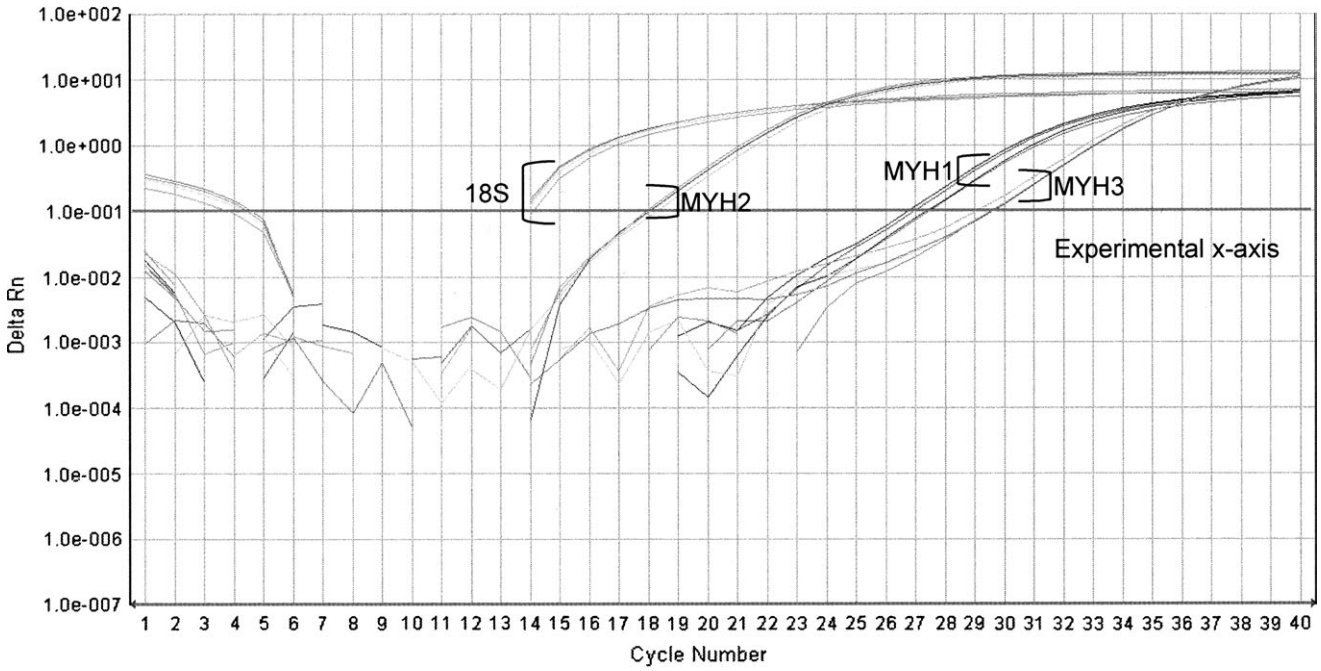

Figure 2 Example of polymerase chain reaction (PCR) amplification curves. Traces were obtained for quadruplicate determinations of 18 S rRNA, *MYH1*, *MYH2*, and *MYH3* expression in the mRNA pool derived from one of the patients in this study. The curves for each quadruplicate are tightly clustered and where they intercept the experimental *x*-axis (cycle number—C_T) is a measure of their relative abundance. In the representative sample shown, the abundance is 18 S > *MYH2* > *MYH1* > *MYH3*. The *y*-axis is a measure of accumulated fluorescence. The 'experimental' *x*-axis is determined by the PCR system below which any signal is not discernable from the background.

experimental technique. In comparing these results with those of other investigators, it must be borne in mind that there are relatively few studies investigating muscle tissue at a cellular or genetic level in humans, and those that do exist are limited due to obvious ethical constraints.

The results of this pilot study have indicated that, despite the small sample size, there are putative differences in MyHC expression in relation to vertical facial morphology. This is particularly evident with regard to the *MYH1* gene that represents the fast MyHC IIx isoform. The results of this investigation confirm previous findings that variations in facial morphology are associated with changes in the expression of this MyHC. Rowlerson *et al.* (2005), using immunostaining techniques, noted a reduction in the contribution of type II fibre phenotypes to masseter muscle structure in subjects with anterior open bites, frequently associated with an increased MM angle. Furthermore, Gedrange *et al.* (2005), using PCR techniques, demonstrated a significant variation in the relative absolute type I and IIx MyHC mRNA expression in patients with horizontal skeletal discrepancies. There is also some evidence of a clustering effect with regard to the embryonic MyHC gene (*MYH3*).

The basis of the application of the present technique was the fact that histological differences in masseter muscle structure are known to exist in patients with different vertical facial forms. It was, therefore, inferred that differences might be present in gene expression between these patients to cause the variations. It is important to appreciate, however, that all the patients had undergone a course of orthodontic treatment prior to surgery and this may have affected the gene expression. Harzer *et al.* (2007), using real-time PCR, noted a shift from type I to type IIa MyHC expression following orthognathic surgery in patients with facial deformity in the horizontal plane. Gene expression depends not only on the presence or absence of a particular gene but also on other variables such as transcription factors or feedback mechanisms.

Analysis of mRNA may be important because it demonstrates which genes are being transcribed by the relevant tissue e.g. its transcriptome [the set of all mRNA molecules, or 'transcripts', produced in one or a population of cells (e.g. a tissue)]. Although various methods of determining the expression of different proteins in tissues exist, the qRT–PCR technique is both extremely sensitive and specific. Importantly, the procedure is an exponential amplification process, so the sample of tissue, and hence RNA, can be very small. This means that smaller muscle biopsies are possible, which is advantageous for the patient and surgeon. In addition, it reveals gene expression at even minute levels, undetectable by other methods. Nucleic acid probes specific to the target genes under investigation are widely available, and both absolute and relative quantitation of the mRNA levels is possible (Mullis *et al.*, 1994). The analysis is also fast and in an automated system could be undertaken in just a few hours.

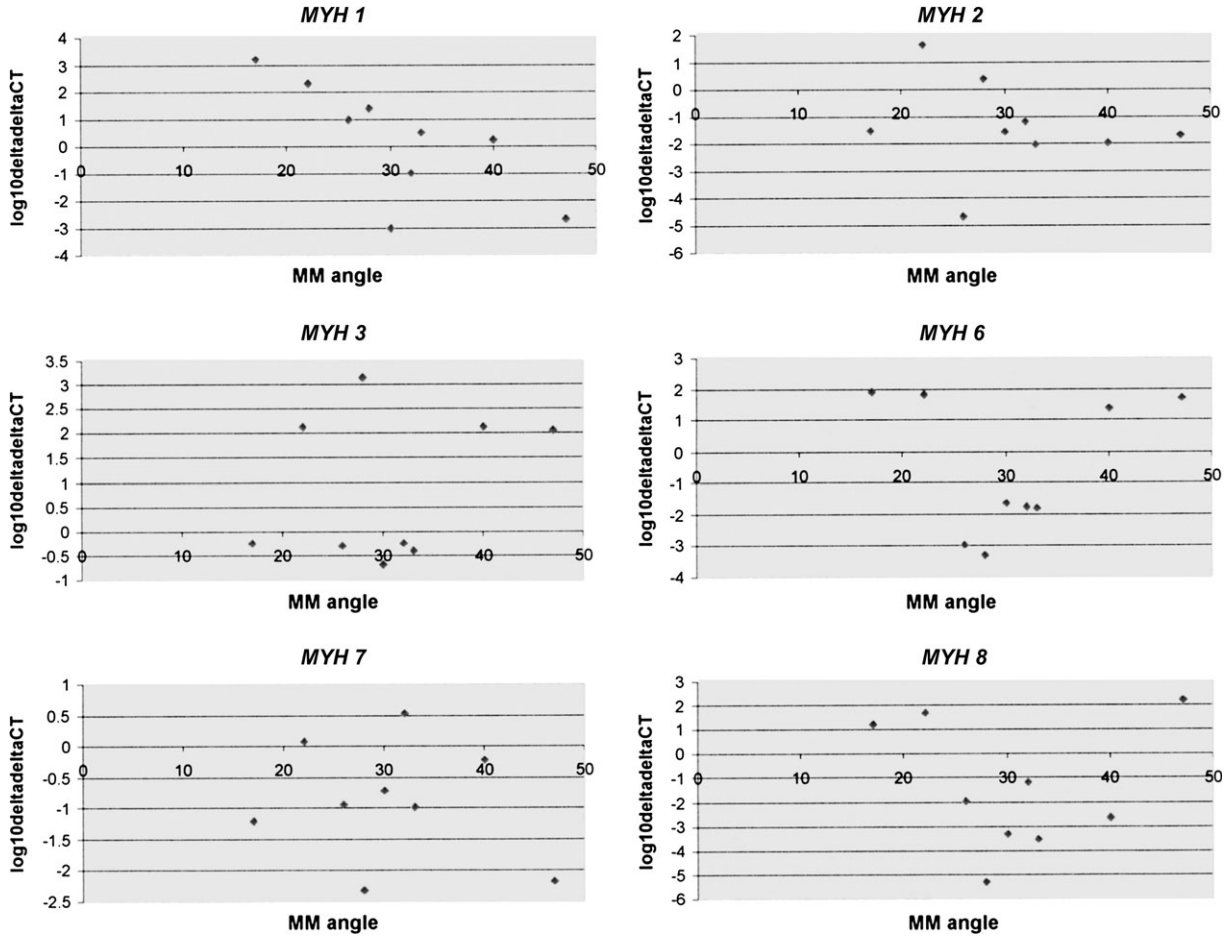

Figure 3 Polymerase chain reaction output comparing levels of genes *MYH1*, *2*, *3*, *6*, *7*, and *8* for the test patients relative to a calibrator patient (with normal vertical dimensions) plotted against maxillo-mandibular value. Relative quantification uses cycle numbers to determine the change (ΔC_T) in expression of a target nucleic acid sequence in a test sample relative to the same sequence in a calibrator sample. The *y*-axis is a derived number from that calculation ($2^{-\Delta\Delta C_T}$).

Although orthodontic diagnosis is largely clinical, it may be supplemented by a number of aids. In the same way that radiographs can help to confirm or refute a clinical impression, the genetic 'fingerprint' of an individual may become another aid to diagnosis and prognosis. Björk (1955) established radiographic indicators of mandibular backward growth rotation, and these are often used to corroborate, for example, the likelihood of an anterior open bite to worsen. If in the same patient, a genetic 'screen' showed high expression of certain muscle genes, also associated with such facial form, this could perhaps also show that the malocclusion was highly likely to worsen. Similarly, other genetic information might help predict muscular adaptive capacity—this might become useful in assessing the prognosis of correction of a malocclusion using functional appliances or orthognathic surgery, both heavily reliant upon muscular adaptation for success.

Clinical practice is increasingly becoming more evidence based. Molecular diagnostic techniques offer an alternative, supplementary approach to patient assessment. Genetic analysis is a well-established tool in medicine. Well-known examples of genes associated with diseases include *BRCA*1, a gene known to be highly penetrant in breast cancer, and *CFTR*, which is the defective gene in cystic fibrosis patients. If certain muscle genes were found to be expressed at consistently high or low levels or found to be defective in excessively long faced or persistent relapse cases, then the potential exists for a test to predict such outcomes. In this way, a myosin molecular fingerprint or profile may identify those with a high probability of incomplete muscular adaptability. The possible implications of the above might mean that the patient is better informed to consent to a particular treatment or that he or she is spared the morbidity of surgery if it is highly likely that the correction will relapse.

Conclusions

It is clear that if the above techniques are to be employed in the future, there needs to be excellent quality evidence upon

which to base any eventual patient tests. It must also be taken into consideration that often multiple genes are involved in the creation of a particular phenotype. This necessitates further research into the genetic basis of facial patterning so that causative genes relating to particular conditions might be identified. A large multicentre database of muscle tissue and patient phenotype could be established, so that sample numbers are high and any statistical tests upon which to make putative associations are more robust. Although this study was constrained by its size, it does illustrate that the use of such technology as a diagnostic aid is a real possibility for the future.

Address for correspondence

Dr Mark P. Lewis
Division of Biomaterials and Tissue Engineering
University College London
Eastman Dental Institute
256 Gray's Inn Road
London WCIX 8LD
UK
E-mail: m.lewis@eastman.ucl.ac.uk

References

Bennington P C M, Gardner J E, Hunt N P 1999 Masseter muscle volume measured using ultrasonography and its relation with facial morphology. European Journal of Orthodontics 21: 659–670

Björk A 1955 Facial growth in man, studied by the aid of metallic implants. Acta Odontologica Scandinavica 13: 9–34

Boyd S, Gonyea W J, Legan H L, Bell W H 1989 Masseter muscle adaptation following surgical correction of vertical maxillary excess. Journal of Oral and Maxillofacial Surgery 47: 953–962

British Standards Classification 1983 British standards glossary of dental terms. BS 4492. British Standards Institution, London, pp. 21–23

Gedrange T, Büttner C, Schnieder M, Oppitz R, Harzer W 2005 Myosin heavy chain protein and gene expression in the masseter muscle of adult patients with distal or mesial malocclusion. Journal of Applied Genetics 46: 227–236

Gedrange T, Luck O, Hesske G, Büttner C, Seibel P, Harzer W 2001 Differential expression of myosin heavy-chain mRNA in muscles of mastication during functional advancement of the mandible in pigs. Archives of Oral Biology 46: 215–220

Gedrange T *et al.* 2006 Change of mRNA amount of myosin heavy chain in masseter muscle after orthognathic surgery of patients with malocclusion. Journal of Cranio-Maxillo-Facial Surgery 34: 110–115

Goldspink G 2003 Gene expression in muscle in response to exercise. Journal of Muscle Research and Cell Motility 24: 121–126

Harzer W, Worm M, Gedrange T, Schneider M, Wolf P 2007 Myosin heavy chain mRNA isoforms in masseter muscle before and after orthognathic surgery. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics 104: 486–490

Hunt N P, Cunningham S J 1997 The influence of orthognathic surgery on occlusal force in patients with vertical facial deformities. International Journal of Oral and Maxillofacial Surgery 26: 87–91

Kiliaridis S 1995 Masticatory muscle influence on craniofacial growth. Acta Odontologica Scandinavica 53: 196–202

Linder-Aronson S 1979 Respiratory function in relation to facial morphology and the dentition. British Journal of Orthodontics 6: 59–71

Lundström A, McWilliam J S 1987 A comparison of vertical and horizontal cephalometric variables with regard to heritability. European Journal of Orthodontics 9: 104–108

Maejima M *et al.* 2005 Changes in the properties of mouse tongue muscle fibres before and after weaning. Archives of Oral Biology 50: 988–993

Mullis K B, Ferre F, Gibbs R A (eds) 1994 The polymerase chain reaction. Birkhauser, Boston

Ohnuki Y, Saeki Y, Yamane A, Yanagisawa K 2000 Quantitative changes in the mRNA for contractile proteins and metabolic enzymes in masseter muscle of bite-opened rats. Archives of Oral Biology 45: 1025–1032

Proffit W R 2000 Contemporary orthodontics, 3rd edn. Mosby, St Louis, pp. 113–143

Proffit W R, Fields H W, Nixon W L 1983 Occlusal forces in normal- and long-face adults. Journal of Dental Research 62: 566–571

Rowlerson A *et al.* 2005 Fiber-type differences in masseter muscle associated with different facial morphologies. American Journal of Orthodontics and Dentofacial Orthopedics 127: 37–46

Talmadge R 2000 Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. Muscle and Nerve 23: 661–679

Weiss A *et al.* 1999 Organisation of human and mouse skeletal myosin heavy-chain gene clusters is highly conserved. Proceedings of the National Academy of Science of the United States America 96: 2958–2963